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PREVALENCE AND GENETIC DIVERSITY OF *C. JEJUNI* ISOLATED FROM BROILERS AND THEIR ENVIRONMENT USING *flaA*-RFLP TYPING AND MLST ANALYSIS

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Abstract

Campylobacter is highly diverse genetically and also undergoes frequent intraspecific recombination. A major source of campylobacteriosis, which is transmitted to humans is found in poultry. The assessment of the genetic diversity among *Campylobacter* population is critical to our understanding of the epidemiology. The genetic diversity of *Campylobacter jejuni* isolates in broilers and their environment were investigated by *flaA*-restriction fragment length polymorphism (RFLP) and multilocus sequence typing (MLST). The study revealed that 92.3% of the examined broiler flocks were contaminated with *Campylobacter* spp. A total number of 35 different *flaA* types defined by *flaA*-RFLP were found in 448 *C. jejuni* isolates originated from broilers, litter, puddles, zones, anteroom and wild birds. The most dominant *flaA* type was XXV. MLST defined 20 sequence types (STs) belonging to 10 clonal complexes (CCs). Among all the STs 9 isolates (15%) were consigned to 2 different STs (ST-7413 and ST-4800), which could not be assigned. The most common CCs were ST-21 and ST-179. The ST-21 CC was common in broilers and environment (puddle water and concentric zones) and the ST-179 CC was specific to wild birds, but also was found in puddle water and concentric zones.

Key words: *Campylobacter jejuni*, poultry, MLST, RFLP, campylobacteriosis

Campylobacter jejuni continues to be the most common cause of bacterial gastroenteritis in the developed world (EFSA, 2015). In the European Union the incidence of campylobacteriosis is 64.8 per 100,000 population (EFSA, 2015). In 2013 in Lithuania campylobacteriosis was the most prevalent food-borne zoonosis in humans with the incidence of 38.3 per 100,000 population (EFSA, 2015) and it was still growing till 2009. Compared with the Baltic States, in Lithuania campylobacteriosis incidence is higher than in Estonia (28.9 per 100,000) or Latvia (0.4/100,000)

(EFSA, 2015). However, in neighboring Poland the incidence of this disease is only 1.4 per 100,000 (EFSA, 2015).

The risk factors of *Campylobacter* infections include the consumption of undercooked meat, contaminated food, water, raw milk and also a direct contact with pets, farm animals and children swimming in lakes and traveling abroad (Jakopanec et al., 2008; Richardson et al., 2007; Ellis-Iversen et al., 2012). But poultry is still a major source of this organism (EFSA, 2015). The investigations in Lithuania showed that broilers' caecal samples had a prevalence of 80.95% (Kudirkienė et al., 2010), broiler wings and drumsticks – 46.55% (Bunevičienė et al., 2010) and cattle – 78.5% (Ramonaitė et al., 2013).

Molecular typing has advanced many epidemiological studies, including the identification of infections of food-borne outbreaks. The molecular epidemiological survey of *C. jejuni* is critical to understand the sources and routes of transmission (Levesque et al., 2008), as well as to develop digression strategies to reduce the incidence of campylobacteriosis (Moffatt et al., 2010). Thus, several methods including MLST (multilocus sequence typing) and *flaA*-RFLP (the restriction fragment length polymorphism analysis of the flagellin-A gene) have been used for epidemiological studies. The MLST has become the gold standard method for studying *C. jejuni* population (Korczak et al., 2009; Larsen et al., 2012). Some *C. jejuni* genotypes isolated from the surrounding environment of certain animals and chickens are found, these vehicles and reservoirs could be a source of contamination for broilers (Ridley et al., 2011).

Due to the increasing number of campylobacteriosis cases in Lithuania and in other countries, it is important to clarify *C. jejuni* population structure and phylogenetic relationships among the samples isolated from broilers and their environment. This study investigated *C. jejuni* population by MLST and *flaA*-RFLP among broilers and their environment over the period of two years. The aim of this study was to determine genetic diversity of *C. jejuni* and investigate the potential relationships between broilers and their environment.

Material and methods

The largest poultry factory in Lithuania has been chosen for the survey. The samples were taken from study house and additional houses during two years' period. The study house is the main poultry house, which was tested from empty house period till the end of broilers growing period. The additional houses were three poultry houses which were near or in front of the study house. In these three houses *Campylobacter* migration was observed.

Flock sampling

The samples from flock were taken once a week from litter (before a broiler placement day and during the raising period of the entire flock). The samples were collected from 13 broiler flocks. To sample a broiler house, one pair of disposa-

ble fabric overshoes (boot socks) was worn over rubber boots as the collector and was used to walk a minimum of 100 meters inside the house. Before use, the boot socks were pre-moistened with sterile physiological saline maximum recovery broth (610077, Liofilchem, Italy) in order to allow the maximum uptake of *Campylobacter* cells from the litter. The boot socks were placed into sterile plastic bag and transported to the laboratory. In case a boot sock was considered positive by PCR, 10 fecal swabs from broiler cloaca were taken to confirm positivity once a week till the first depopulation event. The swabs were placed into tubes with 10 ml sterile modified Exeter broth, which was prepared from Bolton broth (CM985, Oxoid, England) with *Campylobacter* growth (SV61, Mast Diagnostics, Merseyside U.K), *Campylobacter* enrichment (Exeter) (SV59 Mast Diagnostics, Merseyside UK) supplements, and 1% of the lacked horse blood as described previously (Williams et al., 2012). Before slaughter 10 fecal samples were taken for the detection of total *Campylobacter* spp. number and were placed into sterile plastic bags.

Environmental sampling

The samples from the environment were also taken weekly. We took the samples from the zones around the poultry farm, puddles, tap water, flies and feces from rats, wild animals and birds. The environment around the poultry farm was divided into 5 zones (1 m, 40 m, 60 m, 80 m and 100 m). The samples were taken from the boot socks as described above. The samples from the puddles were taken in sterile 100 ml bottles all year round except winter. The tap water samples were taken from a study house and additional houses also in sterile 100 ml bottles, when the broiler age was 20–25 days. Before taking the samples the tap was sterilized and the water was lowered from 3 to 5 minutes. The feces of wild animals, rats and birds were put into sterile bags. Flies were taken with a sterile pincer and put into sterile bags too.

Isolation procedure and identification

Two hundred ml of sterile saline maximum recovery broth (610077, Liofilchem, Italy) was added into the plastic bags with the boot socks taken from the litter and zones and was palpated by hand to release the fecal material. The procedure was used according to the technique of Merga et al. Thus, 10 µl from the same saline with boot socks was streaked onto the *Campylobacter* blood free medium base (mCCDA) (610130, Liofilchem, Italy) with mCCDA Selective Supplement (81037, Liofilchem, Italy). The plates were incubated in a micro-aerophilic atmosphere (5% oxygen, 10% carbon dioxide, 85% nitrogen) generated by Campygen (CN25; Oxoid, UK) at 37°C for 48 h. After the incubation, the colonies suspected of *Campylobacter* were obtained from each plate, examined under a microscope and further purified on the blood agar plates (610188, Liofilchem, Italy) supplemented with 5% lacked horse blood (HBL100, E&O Laboratories, Scotland), and incubated at 37°C for 48 h in the microaerophilic atmosphere. The subcultured isolates were subsequently stored at –80°C in Brain Heart Infusion Broth (BHI) (610008, Liofilchem, Italy) with 30% glycerol (REACHEM, Slovakia) until further use.

Broiler cloacal samples were collected using sterile cotton swabs and directly plated on mCCDA. Plates were incubated in a micro-aerophilic atmosphere at 37°C

for 48 h. The fecal samples from the broilers, which were used for the detection of total *Campylobacter* spp. and also the feces of wild animals, birds and rats were poured on with the modified Exeter broth with proportion 1:10 (1 g of fecal with 10 ml of Exeter broth) and put into the BagMixer for 1 minute. For broiler fecal samples the decimal dilutions up to 10^{-10} were prepared. The *Campylobacter* counts (cfu/g) of the broiler fecal samples were calculated according to ISO 10272–2:2006. One hundred μ l were taken from every dilution and put onto mCCDA agar. Ten μ l from the saline with the feces of wild animals, birds and rats was also streaked onto mCCDA agar. All the plates were incubated in a micro-aerophilic atmosphere at 37°C for 48 h. Each fly was macerated in a sterile mortar, suspended in 2 ml of 0.9% saline. The mixture was centrifuged at 14,000 rpm for 7 min. The pellet was resuspended in 2 ml of Exeter broth and vortexed before the incubation for the enrichment at 37°C for 24 h. After the enrichment, the tube was again centrifuged and 10 μ l was streaked onto mCCDA agar and incubated at 37°C for 48 h in microaerophilic atmosphere. The identification and purification of *Campylobacter* isolates were further performed as described above.

Additionally, a selective enrichment procedure was performed for each sample to detect the low numbers of *Campylobacter* spp. For this purpose, broiler samples swabs were placed into 10 ml modified Exeter broth. Also 1 ml of clear fluid from the saline with boot socks from litter and zones was transferred into 10 ml of Exeter broth for the enrichment. This Exeter broth was prepared from Bolton broth (CM985, Oxoid, England) with *Campylobacter* growth (SV61, Mast Diagnostics, Merseyside UK), *Campylobacter* enrichment (Exeter) (SV59 Mast Diagnostics, Merseyside UK) supplements, and 1% of the lacked horse blood as described previously (Williams et al., 2012). The enrichment tubes were incubated into micro-aerophilic atmosphere at 37°C for 48 h. After the incubation 10 μ l of the broth from other samples (boot socks, feces, puddles, tap water), was streaked onto mCCDA plates. The identification and purification of *Campylobacter* isolates were further performed as described above.

DNA extraction and multiplex PCR

PureLink® Genomic DNA Kit (Life Technologies, Lithuania) was used for the DNA isolation direct from boot socks samples and the presence of *Campylobacter* spp. was confirmed by using PCR assay by Katzav et al. (2008).

DNA isolation was carried out by using GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania). The Supernatant was placed in new tube and stored at –20°C.

Campylobacter isolates were identified to the species level by the modification of the method and the primers as described by Wang et al. (2002) and by Katzav et al. (2008). Primers C412F and CampR2 created a 857 bp fragment, which occurred in all *Campylobacter* spp. A 323-bp amplicon was generated for *C. jejuni* and a 126-bp amplicon was generated for *C. coli* by using a mixture of primers hybridizing to the *C. jejuni* (primers CJF and CJR) and the *C. coli* (primers CCF and CCR). Each PCR mixture contained 2.0 μ l of 2mM deoxynucleotide triphosphates (dNTPs) mixture (Thermo Scientific, Lithuania), 2.5 μ l of 10xPCR buffer, 2.5 μ l of 25 mM MgCl₂, 0.25 μ l of Maxima Hot-Start Taq DNA polymerase 5U/ μ L (Thermo Scientific, Lithu-

ania) and 1.0 µl of a 100 µmol l⁻¹ primer mixture containing C412F, CampR2, *C. jejuni* and *C. coli* primers (Thermo Scientific, Lithuania). PCR products were analyzed by gel electrophoresis: 11 µl of each PCR product was loaded onto 1.9% TopVision Agarose gel (Thermo Scientific, Lithuania) containing 6.5 µl of ethidium bromide solution. The gel was visualized on an UV board. The GeneRuler™ 100 bp DNA Ladder (Thermo Scientific, Lithuania) was used as the molecular size marker.

***flaA* – RFLP genotyping**

The isolates identified as *C. jejuni* were typed by PCR-RFLP for the *flaA* gene. A fragment of 1700 bp of the *flaA* gene was amplified in a PCR reaction by using a pair of specific primers. The PCR was carried out in a 50 µl (final volume) mixture containing 2.5 µl of DNA, 0.5 µl of A1 primer and 0.5 µl of A2 primer, 5 µl of 10xPCR buffer, 5 µl of 2mM dNTP's mix, 3 µl of 25 mM MgCl₂, 0.25 µl of Taq DNA polymerase (Thermo Scientific, Lithuania) and 33.25 µl of sterilized bidistilled water. The samples were first incubated for 1 min at 94°C and then were cycled 35 times at 94°C for 15 s, at 45°C for 45 s, and at 72°C for 1 min 45 s. Afterwards the samples were incubated at 72°C for 5 min and were maintained at 4°C until processed. The PCR product was digested by the incubation at 37°C for 3 h in a reaction mixture containing 21.8 µl of sterile bidistilled water, 3 µl of buffer, 0.2 µl of *Dde*I and 5 µl PCR product. The digested PCR products were run on 2.5% agarose at 90 V for 90 min. The *flaA*-RFLP gels were visualized on UV board and photographed. The images in TIFF format were performed by using BioNumerics version 7.1 (Applied Maths, Belgium). After the normalization of the pattern, the similarity matrix was calculated by using the Dice similarity coefficient and clustering by the Unweighted Paired Group Method with Arithmetic mean values (UPGMA). The band position tolerance and the optimization coefficient were set to 2.0%. The GeneRuler™ 100 bp plus DNA Ladder (Thermo Scientific, Lithuania) was used as the molecular size marker.

MLST typing

The target fragments of the housekeeping genes were *aspA* (aspartase), *glnA* (glutamine synthetase), *gltA* (citrate synthase), *glyA* (serine hydroxyl methyl transferase), *pgm* (phosphor glucomutase), *tkt* (transketolase), and *uncA* (ATP synthase alpha subunit). PCR products were amplified with designed primer pairs. The PCR was carried out in a 25 µl (final volume) mixture containing 2 µl of DNA, 12.5 µl DreamTaq Green PCR Master Mix (Thermo Scientific, Lithuania), 2.5 µl of primer pair mixture and 8 µl of sterile bidistilled water. The reaction conditions were: 9 min at 96°C and then were cycled 30 times at 94°C for 0.5 min, at 52°C for 0.5 min, and at 72°C for 1 min. Later the samples were incubated at 72°C for 7 min and were maintained at 4°C until processed. The amplicons were examined via gel electrophoresis at 120 V 30 min and PCR product was purified using the GeneJET PCR Purification Kit (Thermo Scientific, Lithuania). The purified PCR products were sent to GATC Biotech AG (Cologne, Germany). Allele numbers were assigned for each housekeeping gene, sequence types (STs) and clonal complexes (CCs) by submitting the DNA sequence to the *C. jejuni* MLST database (<http://pubmlst.org/campylobac->

ter). The obtained sequencing data was analyzed with BioNumerics v 7.1 (Applied Maths, Sint-Martens-Latem, Belgium). For this research was selected the most interesting 60 tested isolates of *C. jejuni*. The number of samples for this research was based on RFLP results. The sixty samples of *C. jejuni* were selected for further analysis by MLST from the most common *flaA* types.

Results

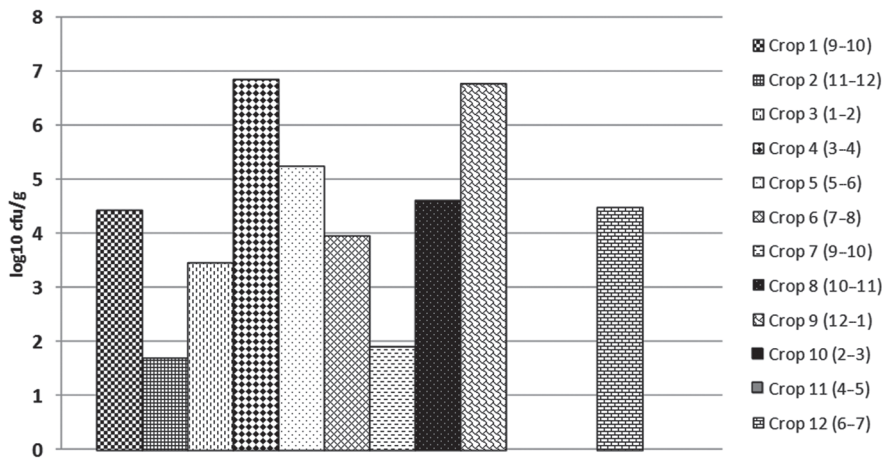
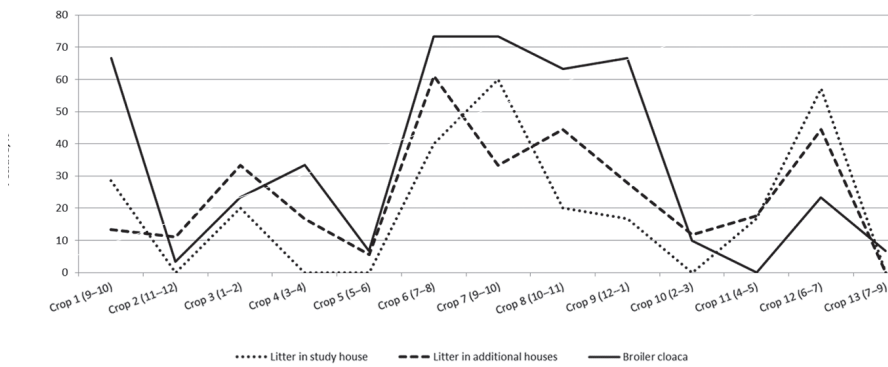
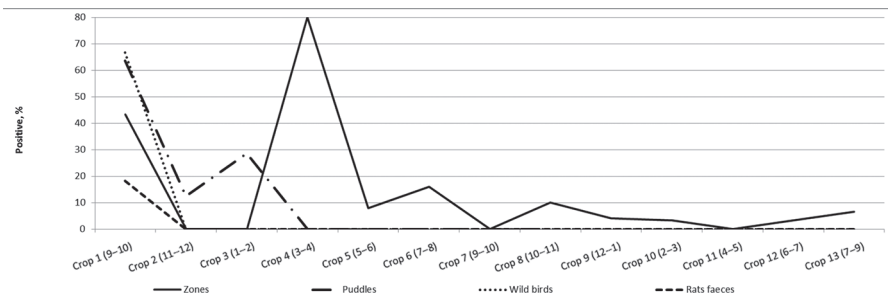
Prevalence of *Campylobacter* in broilers and in environmental sources

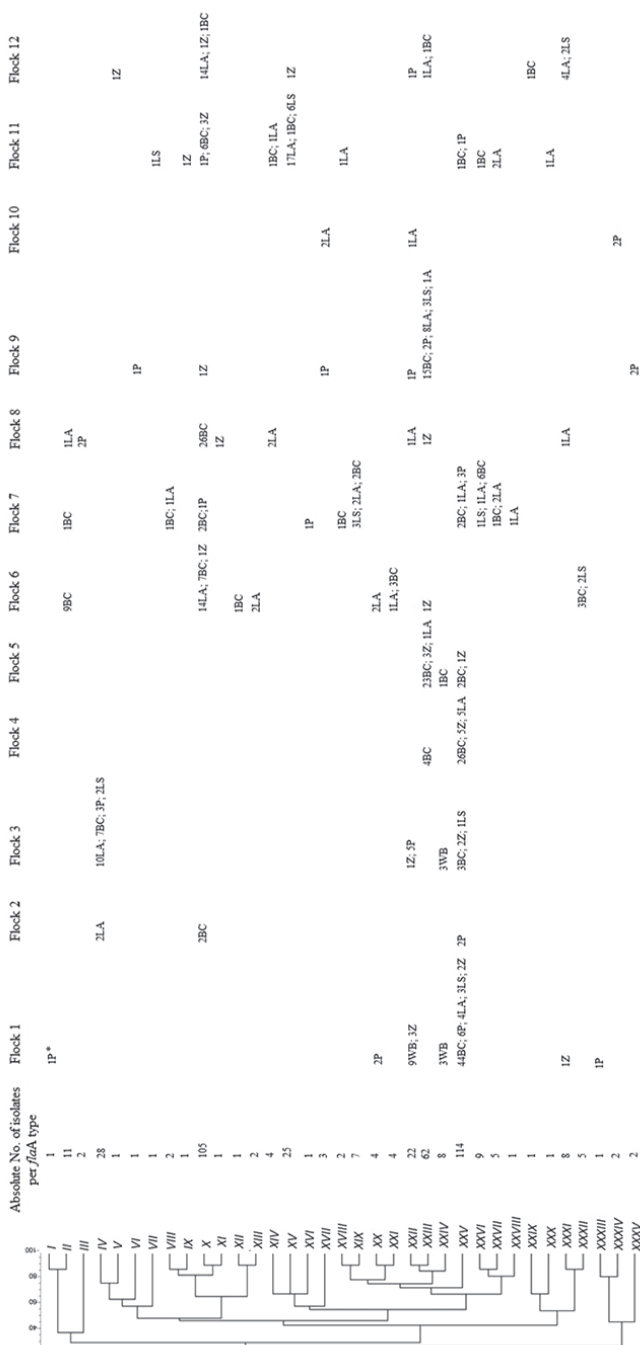
Campylobacter spp. was detected in 12 out of 13 broiler flocks (92.3%). In the period of two years we tested 13 broiler flock rotations and collected 1479 samples. In total, 315 (21.3%) samples were positive for *Campylobacter*. *C. jejuni* was identified in 269 (85.4%) samples and *C. coli* in 26 (8.3%) samples (Table 1). The highest positive samples of *Campylobacter* were found in broiler cloacae, puddle water and in the litter of additional houses (Table 1). The lowest percentage of *Campylobacter* was found in the litter of the study house, concentric zones and wild birds (Table 1). However, a few *Campylobacter* were found in rat fecal samples. We found one positive sample in the anteroom of the study house (Table 1).

Table 1. Prevalence of *Campylobacter* from broilers and farm environment in Lithuania, 2012–2014

The type of source	No. of tested samples	No. (%) of positive samples		
		other <i>Campylobacter</i> spp.	<i>C. jejuni</i>	<i>C. coli</i>
Litter of the study broiler house	81	2 (11.8)	12 (70.6)	3 (17.6)
Litter of additional broiler houses (3)	229	1 (1.8)	42 (77.8)	11 (20.4)
Anteroom of study house	67	0	1 (100.0)	0
Zones	305	0	30 (93.8)	2 (6.2)
Puddles	166	12 (25.5)	35 (74.5)	0
Wild birds	56	0	6 (100.0)	0
Rats	40	2 (100.0)	0	0
Wild animals faeces	12	0	1 (100.0)	0
Flies	138	0	0	0
Broiler cloacae	402	3 (1.9)	142 (91.6)	10 (6.5)
Tap water	50	0	0	0

Campylobacter counts (cfu/g) were calculated before every slaughter of the flock. The data shows that a higher *Campylobacter* spp. number was established in crop 4 during the spring period of March–April (6.86 log₁₀ cfu/g) and in crop 9 during the winter time in December–January (6.78 log₁₀ cfu/g) (Figure 1). The lowest number of *Campylobacter* was in crop 2 (November–December) and crop 7 (September–October).

Figure 1. The numbers of *Campylobacter* spp. before the slaughtering at farmFigure 2. *Campylobacter* spp. prevalence in litter and broiler cloacaFigure 3. *Campylobacter* spp. prevalence in broiler environment



*Source of isolate: LS – litter of study house; LA – litter of additional houses; A – anteroom; Z – zones; P – puddles; WB – wild birds; BC – broiler cloacae.

Figure 4. Prevalence and variety of *C. jejuni flaA* genotypes among different sources

The prevalence of *Campylobacter* in broiler cloaca samples increased in September–October (crop 1) and also from July to August (crop 6 and crop 10) (Figure 2). The results showed the peaking in July–August (crop 6) and in September–October (crop 7), but in April–May (crop 11) no positive samples were found. A higher increase was detected in the litter of study house samples in September–October (crop 7) and June–July (crop 12). In crops 2, 4, 5, 10 and 13 we have not found positive samples. The positivity of the samples from litter of the additional houses showed the increase in July–August (crop 6).

Figure 3 shows positive samples from the environment sources. The highest *Campylobacter* contamination as seen in the samples was collected from the zones, in the puddle and in wild birds. We have to assume that the peak in the zones was reached in March–April (crop 4). The highest prevalence in puddles was in September–October when crop 1 was raised. From March no positive samples from puddles were found. The similar results could be seen in other sources (wild birds and rats feces).

Genotype diversity of *C. jejuni* isolates

The *flaA*-RFLP typing of 448 *C. jejuni* isolates resulted in 35 different *flaA* genotypes (Figure 4). The most frequent *flaA* genotypes were X and XXV, these genotypes were found in the water of puddles, the litter of study and additional houses, broiler cloaca and the zones. Both genotypes were detected in 7 flocks. We distinguished 3 groups of the genotypes. To the first group belong *C. jejuni* genotypes XII and XXVIII and they were dominant just in broiler cloacal samples in flocks 6 and 12. The second group comprises genotypes I, III, V–VII, IX, XI, XIII, XVI, XVII, XIX, XXII, XXIX–XXXI, XXXIII–XXXV which were found just in the farm environment and in the litter of additional houses. And the third group genotypes II, IV, VIII, X, XIV, XV, XVIII, XX, XXI, XXIII–XXVII and XXXII were dominant in all sources.

Diversity and distribution of STs and CCs among broilers and in environmental sources

Among 60 *C. jejuni* isolates which were included in MLST analysis, 20 distinct STs were identified. Among all the STs 9 isolates (15%) were assigned to 2 different STs (ST-7413 and ST-4800), which could not be assigned to any of the known CCs registered at PubMLST database (<http://pubmlst.org/campylobacter>) (Table 2). The most common CCs were ST-21 CC (20%) and ST-179 CC (16.6%). The six most predominant STs (ST-21, ST-4447, ST-257, ST-5, ST-51 and ST-4800) represented approximately half of the isolates (33 out of 60 isolates, or 55%). The ST-21 complex was the most widely distributed clonal complex and was present in 5 of 7 different isolation sources (Table 3). The ST-52 and ST-464 complexes were the least widely distributed. ST-179 complex was predominant in puddles as compared with other CCs. The wild birds had one clonal complex ST-179, which was predominant as compared with other sources. Conversely, unassigned complexes were predominant in broiler cloaca. Two clonal complexes (ST-257 and ST-353) were found in the same sources (the broiler cloaca, the zones and the litter of the study house and ad-

ditional houses). In this study ST-21, ST-257 and ST-353 clonal complexes were common in broiler cloaca, the zones and the litter of all houses. Two sources (the zones and the litter of additional houses) had 8 out of 11 different clonal complexes and six clonal complexes were common to both isolation sources. The population structure of the isolates was also evaluated with a minimum-spanning tree (Figure 5) using the allelic data with the program Bionumerics V 7.0. The phylogenetic analysis showed that the most STs are common in various sources. Only few STs are specific and found in these sources, such as ST-464 established from anteroom; ST-5013 and ST-2066 established from the litter of additional houses and ST-7413 and ST-5777 established from the broiler cloaca.

Table 2. Distribution of 60 *C. jejuni* isolates among CCs and STs

CC	ST	No. of <i>C. jejuni</i> isolates
21	19	1
	21	6
	376	3
	2857	2
48	429	3
52	2066	1
179	220	2
	2209	2
	4447	6
257	257	5
	2254	2
353	5	5
	5011	1
443	51	6
464	464	1
607	607	2
828	829	2
	5777	1
Unassigned	7413	4
	4800	5

Table 3. Frequency and distribution of clonal complexes among broilers and their environmental sources of *C. jejuni*

Source (n)	Clonal complex										
	21	48	52	179	257	353	443	464	607	828	Unassigned
Broiler cloacae (17)	4	–	–	–	2	1	1	–	–	2	7
Puddles (7)	2	1	–	3	–	–	1	–	–	–	–
Wild birds feces (5)	–	–	–	5	–	–	–	–	–	–	–
Zones (11)	1	–	–	2	2	1	2	–	1	1	1
Litter of the study house (6)	3	–	–	–	1	1	–	–	–	–	1
Litter of additional houses (13)	2	2	1	–	2	2	2	–	1	1	–
Anteroom of study house (1)	–	–	–	–	–	–	–	1	–	–	–

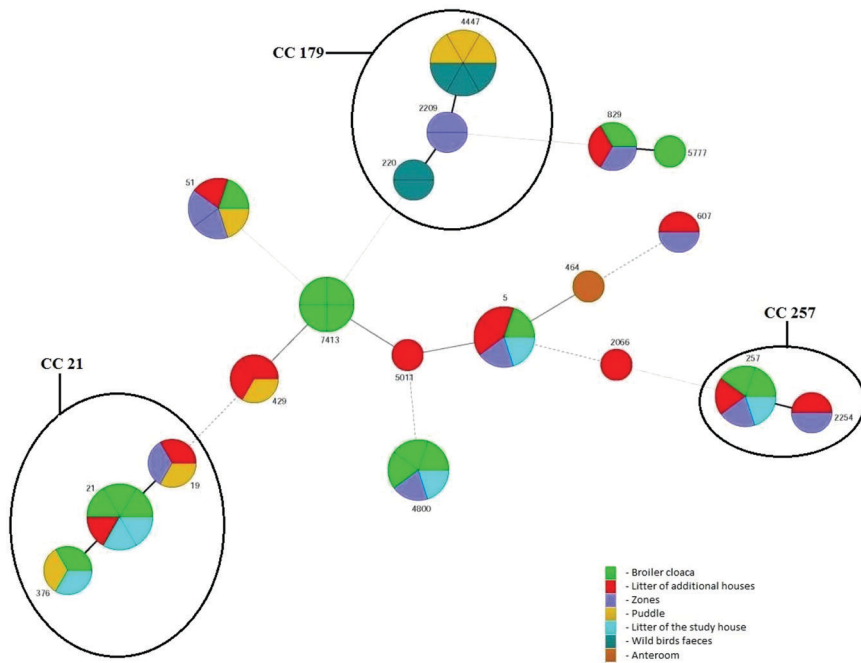


Figure 5. Phylogenetic analysis of *C. jejuni* isolates from broilers and their environment by MLST. Each circle and number by the circle represents one ST: circles of increased diameter represent higher strain numbers within one ST, different colours indicate strain source. Numbers in bold by the circle represent three of the most prevalent CCs revealed in this study

Discussion

The horizontal transmission from the environment is considered to be the most frequent source of *Campylobacter* spp. to broilers. It is widely accepted that the horizontal transmission within a flock occurs rapidly in case individual birds are recolonized by *Campylobacter* (Carrillo et al., 2004; Horrocks et al., 2009). Once established it is very difficult to eliminate. High flock size, environmental water supplies, litter, insects, wild birds, rodents, fecal contact, personnel and other animals, may increase the risk of colonization and dissemination (Adkin et al., 2006; Horrocks et al., 2009).

This study shows the higher prevalence (92.31%) of *Campylobacter* spp. in broiler flocks compared to the previous study of Kudirkienė et al. (2010). In neighboring Poland also most of the flocks were positive (96.5%) (Wieczorek and Osek, 2015). Furthermore, the investigation as reported by Hungary and the United Kingdom showed very high proportions of positive samples (from 74.2% to 80%) (EFSA, 2015) as well. Our study has revealed that the environmental sources carrying *Campylobacter* in the poultry farm were associated with positive broiler flocks. Fi-

figure 2 shows that *Campylobacter* prevalence in broilers and in the litter are related. If the litter is positive to *Campylobacter* then the flock is positive too. The higher prevalence of *Campylobacter* in litter and in broilers was found from July to January. Dry and aerobic conditions of clean fresh litter are considered harmful to *C. jejuni* as reported by Newell and Fearnley (2003) and Hutchinson et al. (2005). On the other hand, litter can be contaminated by broiler fecal droppings, which favors pathogen transmission through the flock. Dirty contaminated litter can spread in the poultry farm and scatter the microorganism in the environment.

This study has showed that in the period of September–October (crop 1) high *Campylobacter* spp. prevalence was found in wild birds (66.6%), however, from December we did not find any positive samples. In contrast, other studies showed the high *Campylobacter* spp. frequency in wild birds (Brown et al., 2004; Hughes et al., 2009). In general, *Campylobacter* can survive in water, for this reason puddles were found to be positive, but just from September to April. Later we did not detect positive samples. Maybe the positivity of puddles may reflect the general level of cleanliness and contamination of other environmental sources in the farm.

The typing of *Campylobacter* isolates from broilers and their environment provides epidemiological information which is needed for the infection control and the transmission. *flaA*-RFLP typing and MLST were used to explain genetic diversity among isolates.

Results from *flaA*-RFLP typing showed that most of the genotypes were the same in broilers and in their environment. These genotypes were found first in the environment, later in the litter and next in the broilers. Such phenomenon indicates that the bacteria move from the environment to the flock. There are several potential sources of *Campylobacter* that show a temporal correlation with the flock becoming *Campylobacter* positive, such as rodents (Umali et al., 2012; Nkogwe et al., 2011), humans (Messens et al., 2009; Ridley et al., 2011) and water (Patriarchi et al., 2011), which may indicate that these potential sources are actually becoming contaminated by the broiler flock as it is colonized. Catching crews and independent operators (Young et al., 2010) were identified as a potential source of new genotypes in the flock during late grow-out when *Campylobacter* is shedding in the contaminated flock peaks in this way increasing the chances of within-farm and industry-wide *Campylobacter* dissemination. During the analysis we found only one positive sample from the anteroom in flock 9 and it belongs to XXIII *flaA* type. The contamination of the anteroom floor may actually suggest the possible infection of the flock by the farmer, visitors and equipment. Unsuitable hygiene practices at the farm level, especially poor cleaning and disinfection of the house and not dedicated protective clothing, could be a major reason of *Campylobacter* contamination persistence in poultry flocks as was stated by Allen and Newell (2005). Two flocks (7 and 11) had 10 different genotypes, which were found in broilers and in their environment. These flocks were raised in different seasons, flock 7 was raised in April–May and flock 11 was raised in the period of August–September. However, both seasons had the same temperature (7–16°C). In flocks 2, 3, 4, 9, 10 we found less different genotypes. All these flocks were raised in winter and in spring and the temperature was below 0°C. The conclusion can be made that the temperature has the effect on *Campylobacter*

survival mechanism. Many studies showed that *Campylobacter* is sensitive to low temperatures (Silva et al., 2011; Vashin and Stoyanchev, 2011) and, therefore, has less chances to survive in the environment and spread to the flocks. A similar pattern with high diversity of types in warm season and lower diversity of types in cold season has been reported in other studies as well (Hiatt et al., 2002; Kudirkienė et al., 2010). The most dominant is *flaA* type XXV, which was found in 7 flocks out of 12. These flocks were raised in different seasons (spring, autumn and winter). We could assume that this *flaA* type is more tolerant to the environmental stresses. Duffy et al. (2015) and Allen et al. (2007) also suggested that some genotypes could be more tolerant to the environmental stresses in poultry processing.

MLST is an important technique which was invented to study population of *Campylobacter* spp. (Dingle et al., 2001; Levesque et al., 2008). Molecular typing has previously been used to infer the source of campylobacteriosis and the overlap in the genotypes of *Campylobacter* species present in humans, broilers and other sources (Revez et al., 2014; Ramonaitė et al., 2014; Llarena et al., 2014; Zhang et al., 2015). In the study for MLST analyses the isolates have been selected randomly. The isolates were selected from *flaA* types, which were isolated from various sources. 8 isolates were selected from IV *flaA* type, 1 – from V *flaA* type, 6 – from VII *flaA* type, 7 – from VIII *flaA* type, 1 – from IX *flaA* type, 2 – from X *flaA* type, 3 – from XI *flaA* type, 2 – from XII *flaA* type, 5 – from XVI *flaA* type, 3 – from XVIII *flaA* type, 5 – from XX *flaA* type, 3 – from XXI *flaA* type, 2 – from XXIV *flaA* type, 2 – from XXV *flaA* type, 8 – from XXIX *flaA* type, 1 – from XXXII *flaA* type and 1 – from XXXIII *flaA* type. We analyzed the ST diversity and the population structure of 60 *C. jejuni* isolates from different sources. Twenty STs were defined from 60 isolates, which were classified into 10 clonal complexes. Many previous studies have shown that the main clonal complex varies in different regions and countries but CCs, such as ST-45, ST-21, ST-48 and ST-353 are dominant STs that contain the largest number of isolates (Petkovic et al., 2009; Ramonaitė et al., 2014; Zhang et al., 2015). In the study out of 10 identified CCs, five (ST-21 CC, ST-179 CC, ST-443CC, ST-353 CC and ST-257 CC) were the most prevalent and 41 (68.3%) of our examined isolates were labelled to these clonal complexes. At least three individual isolates were assigned to one of these five clonal complexes. One of these clonal complexes (ST-179 CC) was specific to wild birds and 5 *C. jejuni* isolates (8.3%) were labelled to this clonal complex. ST-21 CC was dominant in broilers (6.7%) and in the litter of the study house (5%). All these clonal complexes were identified from different sources: 12 (20%) of the tested isolates were assigned to ST-21 CC, 10 (16.7%) to ST-179 CC, 7 (11.7%) to ST-257 CC, 6 (10%) to ST-353 CC and 6 (10%) to ST-443 CC. The remnant five clonal complexes were identified sporadically among tested *C. jejuni* isolates.

Some studies have reported that *C. jejuni* strains which belong to the ST-21 complex can colonize a multiplicity of hosts and they are one of the most common CCs that infect humans (Cody et al., 2013; El-Adawy et al., 2013; Ramonaitė et al., 2014; Zhang et al., 2015). Thus, the study showed that ST-21 CC contains four strains from broilers, three strains from the litter of the study house, two strains from the puddle and the litter of additional houses and 1 strain from the zones. This clonal complex

is associated with poultry and environment. A similar pattern has been reported in other studies (de Haan et al., 2010; Magnusson et al., 2011; Zhang et al., 2015) but in Ramonaitė et al. (2014) study they have not identified ST-21 CC among *C. jejuni* isolates from environmental sources.

ST-179 CC was the most common complex in the feces of wild birds, also this complex was found in the puddle and in the zones (environment). The prevalence of this clonal complex in wild birds and the puddle was previously registered by Ramonaitė et al. (2014) and Meinersmann et al. (2013). In our study three STs (220, 2209, 4447) assigned to this complex were identified. According to the PubMLST these STs are found in human stool, sand, environmental water, wild birds and in chickens.

ST-257 and ST-353 clonal complexes are also described in other studies (Colles et al., 2003; Zhang et al., 2015; El-Adawy et al., 2013). We assigned two STs (257, 2254) to ST-257 complex and one ST (5011) to ST-353 complex, all strains were isolated from broilers, litter and zones. Interestingly, these STs according to PubMLST are found in human stool, turkey, chicken meat, but not in environmental sources.

According to PubMLST ST-51 of ST-443 complex is found in various sources, such as dog, duck, cattle, human stool, chicken meat and turkey. In our study, we have found ST-51 in the broiler, the puddle, the zones and in the litter of additional houses. However, in other studies this clonal complex was found sporadically (Zhang et al., 2015; Ramonaitė et al., 2014).

The results of our study indicate that *Campylobacter* spp. is widespread in broilers and in environmental sources, with the variation of genotypes. In conclusion, the most important thing is to prevent *Campylobacter* spread in the environment to enter the broiler house. We found STs among *C. jejuni* isolates from the litter, wild birds, the zones and the puddles, thus, the fact reveals that these *Campylobacter* sources are not enough investigated. Further studies are needed to analyze all possible sources which induce the spread of *Campylobacter*. Furthermore, isolates have to be characterized by genome sequencing for a better understanding of campylobacteriosis epidemiology.

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